

■ **ARTHRITIS**

# Activation of the leptin pathway by high expression of the long form of the leptin receptor (Ob-Rb) accelerates chondrocyte senescence in osteoarthritis

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**Objectives**

Activation of the leptin pathway is closely correlated with human knee cartilage degeneration. However, the role of the long form of the leptin receptor (Ob-Rb) in cartilage degeneration needs further study. The aim of this study was to determine the effect of increasing the expression of Ob-Rb on chondrocytes using a lentiviral vector containing Ob-Rb.

**Methods**

The medial and lateral cartilage samples of the tibial plateau from 12 osteoarthritis (OA) patients were collected. Ob-Rb messenger RNA (mRNA) was detected in these samples. The Ob-Rb-overexpressing chondrocytes and controls were treated with different doses of leptin for two days. The activation of the p53/p21 pathway and the number of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal)-positive cells were evaluated. The mammalian target of rapamycin (mTOR) signalling pathway and autophagy were detected after the chondrocytes were treated with a high dose of leptin.

**Results**

In total, 12 cases were found to have severe medial cartilage wear compared with the lateral cartilage. Immunofluorescence showed that the expression of Ob-Rb in the medial cartilage of the tibial plateau was high. High levels of leptin led to cell cycle arrest and inhibited autophagy. After overexpression of Ob-Rb, the physiological dose of leptin induced cell senescence in the chondrocytes. High doses of leptin inhibited autophagy by activating the mTOR signalling pathway. Blockade of the mTOR signalling pathway could restore autophagy and partially reverse senescence induced by leptin in chondrocytes.

**Conclusion**

In summary, the present study demonstrated that high doses of leptin induce cell senescence by activating the mTOR pathway in chondrocytes from OA cartilage. Highly expressed Ob-Rb accelerates chondrocyte senescence by activating the leptin pathway in OA.

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**Keywords:** Osteoarthritis, Ob-Rb, Chondrocyte, Leptin, Autophagy, Senescence

**Article focus**

- This study investigated the role of the long form of the leptin receptor (Ob-Rb) in the development of osteoarthritis (OA), as well as the potential mechanisms involved.
- High doses of leptin inhibited autophagy by activation of the mammalian target of rapamycin (mTOR) signalling pathway in chondrocytes.

**Key messages**

- Degenerative cartilage was found to have a high expression of Ob-Rb.
- After overexpression of Ob-Rb, the physiological dose of leptin induced cell senescence in chondrocytes.

- Blocking the mTOR signalling pathway could restore autophagy and partially reverse senescence induced by leptin in chondrocytes.

**Strengths and limitations**

- These findings may provide the basis for research into increased mechanical stress.

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- Further animal studies of leptin receptor knockout are necessary to establish a better understanding of the pathogenesis of OA.

## Introduction

The main feature of knee osteoarthritis (OA) is cartilage degeneration.<sup>1</sup> Obesity may lead to cartilage degeneration by increasing the mechanical stress across the knee joints and by promoting the production of adipokine by white adipose tissue in overweight people.<sup>2-4</sup> The first identified adipokine, leptin, is likely to be an important mediator linking obesity and OA.<sup>5,6</sup> One study found that the proteoglycans in articular cartilage were depleted when leptin was injected into the stifle joints of rats.<sup>7</sup> Furthermore, another study found that cartilage treated with small interfering RNA (siRNA)-leptin from OA patients showed decreased matrix metalloproteinase (MMP)-13 expression.<sup>8</sup> We found that leptin changes the differentiation fate and induces senescence by the p53/p21 pathway in chondrogenic progenitor cells (CPCs).<sup>9</sup>

Leptin is closely related to the progression and severity of OA. We found that the degree of cartilage degeneration is uneven in the same OA patient (the medial cartilage from the tibial plateau and the femur are significantly degenerated).<sup>9</sup> Interestingly, however, the effects of the patient's joint fluid on the medial and lateral cartilage remained consistent, and we speculated that this may be due to the high expression of the long form of the leptin receptor (Ob-Rb) in degenerative cartilage.<sup>9</sup> The study verified this hypothesis and found that cartilage is severely degenerative when there is a high expression of Ob-Rb.<sup>9</sup> This led us to investigate whether abnormally expressed Ob-Rb mediates the leptin pathway activation and promotes leptin-induced cartilage degeneration.

The inhibition of leptin pathway activation is an important means of intervention in OA. Studies have shown that early inhibition of Ob-Rb expression can prevent the progression of cartilage degeneration caused by mechanical stress in animal OA models.<sup>10</sup> Given the decreased cell viability in chondrocytes induced by leptin in OA patients, reversing the inhibitory effect of leptin on cell viability is a therapeutic target. Leptin can cause degeneration of cartilage by several key pathways, and reversing cell ageing is an important intervention target. How to prevent cartilage from being destroyed as a result of leptin-induced senescence remains to be seen. Studies have shown that autophagy may be related to the pathogenesis of OA and can protect human chondrocytes from stress.<sup>11,12</sup> During the damaging process of OA, the expression of Beclin1 and LC3 is decreased in cartilage. Decreased autophagy in OA contributes to chondrocyte death and cartilage damage.<sup>11,13</sup>

This study explored the effects and possible mechanisms of autophagy in leptin-induced senescence during OA pathogenesis.

## Materials and Methods

**Osteoarthritis cartilage collection.** Osteoarthritis cartilage samples were obtained from 12 patients (aged 55 to 65 years) who underwent total knee arthroplasty for clinically and radiologically diagnosed OA. The patients were excluded if they presented with a history of rheumatic arthropathies or infection in the knee. All tissues were obtained with fully informed consent and prior institutional ethical approval. All samples were harvested from the major load-bearing areas on the medial tibial plateau (MTP) and lateral tibial plateau (LTP).

**Isolation of chondrocytes.** Articular cartilage was obtained from the major load-bearing areas of the LTP from an OA knee, as much of the cartilage tissue in the force area was removed so that more chondrocytes could be obtained after digestion. The cartilage was harvested and subjected to sequential trypsin/collagenase digestion in order to isolate the chondrocytes, as previously described.<sup>9</sup> Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York), penicillin/streptomycin (50 000 U/50 mg), and L-glutamine (4.5 mM), was used to expand the chondrocytes. The cells were cultured under standard conditions. To avoid interference caused by dedifferentiated chondrocytes, we used two to three passage cells.

**Overexpression of Ob-Rb in the chondrocytes.** The green fluorescent protein (GFP)-expressing lentiviral particles were produced by cotransfection with lentiviral plasmids, psPAX2 and pMD2.G (Shanghai Genechem, Shanghai, China). The Ob-Rb-expressing lentiviral particles were produced by the cotransfecting plasmids pCDH-Ob-Rb, psPAX2, and pMD2.G (Shanghai Genechem). The cells at passage 2 with 60% to 70% confluence were used for transduction. After grouping, the cells were transduced with lenti-GFP or lenti-Ob-Rb at a multiplicity of infection (MOI) of 50 for ten hours. After transduction, the cells were exposed to 4 µg/ml puromycin (Sigma-Aldrich, St. Louis, Missouri) for one day in order to obtain stable transduction. The cells transfected with the only GFP-expressing lentiviral vector were used as controls. Cells were used for further experiments after amplification.

**Autophagic flux measured by flow cytometry.** The monomeric red fluorescent protein (mRFP)-enhanced green fluorescent protein (eGFP)-LC3 lentiviral vectors (mRFP-eGFP-LC3) were provided by Shanghai Genechem. The lentivirus was transfected into chondrocytes according to the manufacturer's protocol at a MOI of 50 for six hours. After grouping, lentiviral-transfected chondrocytes were used for subsequent experiments. After completing the corresponding experiment, each group of cells was digested with trypsin to prepare a cell suspension. The mRFP and eGFP signals were analyzed by flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lakes, New Jersey). Autophagic flux of chondrocytes was measured according to a protocol described by Gump and

Thorburn.<sup>14</sup> The autophagic flux was calculated by quantifying the ratio of mRFP to eGFP by Flowing Software (created by Perttu Terho, University of Turku, Finland; <http://flowingsoftware.btk.fi>).

**Immunohistochemical analysis.** Knee cartilage from the major load-bearing areas on the MTP and LTP was subjected to immunohistochemical analysis (IHC) using antimammalian target of rapamycin (mTOR) antibodies (Abcam, Cambridge, United Kingdom). Briefly, the cartilage was deparaffinized and rehydrated and then subjected to antigen retrieval by incubating the tissues in sodium citrate buffer (0.01 M, pH 6.0) at 95°C for ten minutes. The tissue sections were exposed to hydrogen peroxide (3% H<sub>2</sub>O<sub>2</sub>) for five minutes to quench the endogenous peroxidase and were then blocked in 30% horse serum for 30 minutes. The slides containing the tissue sections were incubated overnight at 4°C with primary mTOR antibodies (1:150 dilution). Nonimmune mouse immunoglobulin G (IgG) was used as a negative control. After the tissues were washed with 1 × tris-buffered saline containing 0.1% Tween-20 (TBST), the slides were then incubated with biotinylated secondary antibodies (antigoat IgG; Santa Cruz Biotechnology, Santa Cruz, California) and detected using an avidin-biotin complex (ABC) kit (Vector Laboratories, Burlingame, California).

**Immunofluorescence.** To immunostain the cells or tissues (frozen sections), the samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature. After the cells were washed three times in PBS/0.1% bovine serum albumin (BSA) for five minutes, they were permeabilized using 0.2% Triton (T9284; Sigma-Aldrich) in PBS for 20 minutes and then washed in PBS/0.1% BSA. Primary antibodies against Ob-Rb (Abcam) were diluted in PBS/0.1% BSA and incubated overnight at 4°C. After the samples were washed, the cells or frozen sections were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat antirabbit secondary antibody (1:500; Abcam), a goat antimouse IgG secondary antibody (1:500; Abcam), and 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for one hour at room temperature. Fluorescence images were obtained using a Nikon Eclipse TE2000-U inverted fluorescent microscope (Nikon, Tokyo, Japan).

**Effects of leptin on chondrocyte proliferation.** The chondrocytes were seeded in 96-well plates at a density of 10<sup>4</sup> cells/ml. Cell viability was measured by the Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Rockville, Maryland). After treatment for the indicated times, the culture medium was changed to fresh medium with 10% CCK-8 reagent. After incubation for two to four hours, the absorbance at 450 nm was measured by a microplate reader. Each treatment group had three replicates.

**Cell cycle analysis.** The cell cycle assay was performed using a cell cycle and apoptosis kit (Beyotime Institute of Biotechnology, Shanghai, China). Cells (1 × 10<sup>6</sup> cells

per sample) were collected and passed through a 40 μm nylon membrane (Corning Inc., Corning, New York). Cold 75% alcohol (1 ml) was added to the cells for 24 hours at 4°C, and the cells were then resuspended in 250 μl of propidium iodide (PI) solution/0.1% Triton X-100/RNase A (Beyotime Institute of Biotechnology), incubated at room temperature for 30 to 45 minutes, and analyzed by flow cytometry (BDBiosciences, San Jose, California) for cell cycle analyses.

**Senescence-associated β-galactosidase staining.** Briefly, the cells were washed twice with PBS, fixed in the wells using 1 ml fixing solution per well, and incubated at room temperature for 15 minutes. Following washes with PBS, the cells were incubated in a freshly prepared senescence-associated β-galactosidase (SA-β-gal) staining solution at 37°C, without CO<sub>2</sub> and protected from light, for 24 hours (Cell Signaling Technology, Danvers, Massachusetts). Senescent cells were determined by counting the number of blue-stained cells under a BX40 Olympus microscope (Olympus America, Miami, Florida) equipped with a Nikon N2000 camera (Nikon, Melville, New York). The total number of cells and the number of β-gal-positive cells were determined for five fields of view (200× magnification) per sample. The total number of cells was independently quantitated, and the percentage of senescent cells was calculated accordingly.

**Cell signalling studies.** The chondrocyte cultures were treated with 200 ng/ml leptin (598-LP-05M; R&D Systems, Minneapolis, Minnesota), rapamycin (Sigma-Aldrich), or AZD (Sigma-Aldrich).<sup>15</sup> The cells were cultured in DMEM supplemented with 5% FBS, penicillin/streptomycin (50 000 U/50 mg), and L-glutamine (4.5 mM). After 48 hours of treatment, p-S6 kinase (S6K), p53, p21, and LC3-II (all Abcam) were detected using Western blot analysis. At 48 hours after treatment, cell senescence was evaluated using a commercial staining kit. SA-β-gal-positive cells were counted in five visual fields using light microscopy (200× magnification).

**Western blot analysis.** All cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing a cocktail of protease and phosphatase inhibitors (Beyotime Institute of Biotechnology) at 4°C for one hour. The cell lysates were centrifuged at 13 000 × g and 4°C for 20 minutes, and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein were heated to 100°C for five minutes, separated by 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, California). The membranes were blocked with TBST containing 5% nonfat dried milk for one hour and incubated with primary antibodies overnight at 4°C. The membranes were washed three times with TBST and then incubated with

**Table I.** Age, sex, and body mass index (BMI) of 12 patients with osteoarthritis

Patient number	Age, yrs	Sex	BMI, kg/m <sup>2</sup>
1	64	Male	26.7
2	59	Female	27.8
3	56	Female	29.7
4	55	Male	25.9
5	62	Female	24.1
6	58	Male	28.3
7	61	Female	28.8
8	59	Female	30.6
9	57	Female	27.4
10	65	Male	26.4
11	59	Female	28.7
12	62	Female	26.2

horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. After three additional washes with TBST, the signal intensity was quantified with a ChemiDoc-It Imaging System (UVP, Upland, California) attached to a BioChem HR camera (UVP). To measure protein abundance, the grey values of the blots in the scanned images were measured using ImageJ Plus software (National Institutes of Health, Bethesda, Maryland). Before comparisons were made, the grey value of each target protein was normalized to the value for  $\beta$ -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Safranin-O and Fast Green staining.** The knee OA cartilage samples ( $n = 24$ ) from the major load-bearing areas in the medial and lateral cartilage of the tibial plateau were cut into two to three pieces by bone saw. The specimens were fixed with 4% paraformaldehyde, decalcified with 10% ethylenediaminetetraacetic acid (EDTA; pH 7.4), and embedded in paraffin, followed by dehydration with serial ethanol and clearance with xylene. Sections 5  $\mu$ m thick were then cut, dewaxed, and hydrated with serial ethanol, then stained with Safranin O and Fast Green for histological grading, as described by Mankin et al.<sup>16</sup> The Mankin scoring system contains four indicators: structure (seven grades), cell (four grades), Safranin O staining (five grades), and tidemark (two grades).

Two blinded observers (ZL and GL) scored for histological grading using the Mankin scoring system.

**Statistical analysis.** For detecting Ob-Rb-mRNA expression, the reverse transcriptase polymerase chain reaction (RT-PCR) experiment was repeated three times. We used the OA 3, OA 5, OA 8, and OA 11 specimens from which to harvest and culture chondrocytes from the LTP cartilage. The results from the *in vitro* experiments were analyzed using one-way analysis of variance (ANOVA) or Student's *t*-tests if only two conditions were being compared. All data were analyzed using Prism v. 5.0b software (GraphPad Software, San Diego, California).  $p$ -values  $\leq 0.05$  were considered statistically significant. The results are expressed as the mean and standard deviation.

## Results

### Degenerative cartilage has a high expression of Ob-Rb.

Leptin bioactivity is mediated by specific leptin receptors. At least six types of leptin receptors have been found in mammalian cells. However, the long isoform of Ob-Rb is the functional receptor and plays a major role in signal transduction.

Although 40% of the elderly population have symptoms of knee cartilage degeneration, individuals' cartilage tissue degeneration is usually uneven. Using the characteristics of uneven degeneration in OA cartilage, we conducted a self-controlled study that compared the medial cartilage (degenerated cartilage) with the lateral cartilage (as non-OA-affected region) from the tibial plateau in the same OA patients to assess whether Ob-Rb is highly expressed in degenerated cartilage tissue. We collected medial and lateral cartilage samples of the tibial plateau from 12 OA patients (four male, eight female). The details of the patients, including sex, age, and body mass index (BMI), are presented in Table I. In addition, the degree of degeneration of the medial and lateral cartilage of the tibial plateau was assessed using Safranin O and Fast Green staining (Fig. 1a). In all 12 cases, medial cartilage wear was more severe than lateral cartilage wear (Fig. 1b). Immunofluorescence results showed that the expression of Ob-Rb in the medial cartilage of the tibial plateau was high (Fig. 1c). The results from RT-PCR also confirmed that the expression of Ob-Rb in the medial cartilage of the 12 OA patients was high (Fig. 1d).

High doses of leptin induce cell cycle arrest and senescence in chondrocytes

Although we previously observed leptin-induced cell senescence in CPCs from knee cartilage in Sprague Dawley rats, whether this phenomenon exists in human knee chondrocytes needs to be verified. The physiological concentration of leptin in humans is approximately 10 pg/ml;<sup>17</sup> however, *in vitro* and human body conditions are different. We therefore treated the chondrocytes with the following doses of leptin: 0 ng/ml as control; 10 ng/ml as a physiological dose; and 100 ng/ml and 200 ng/ml as high doses. We explored the effects of different doses of leptin (0 ng/ml, 10 ng/ml, 100 ng/ml, and 200 ng/ml) on chondrocyte proliferation using the CCK-8 reagent and cell cycle analyses. Treating the cells with high doses of leptin resulted in less proliferation than that observed when the cells were treated with the control or physiological doses, and leptin treatment induced cell cycle arrest in the chondrocytes by inhibiting the G1/S cycle and decreased the cell proliferation rate by reducing the (S+G2)% (Figs 2a and 2b). Cell cycle arrest generally leads to quiescence or senescence.<sup>18</sup> Treating the cells with 100 ng/ml and 200 ng/ml leptin resulted in a higher percentage of SA- $\beta$ -gal-positive chondrocytes than that observed in the cells treated with the control or physiological dose of leptin (Fig. 2c). The high doses of

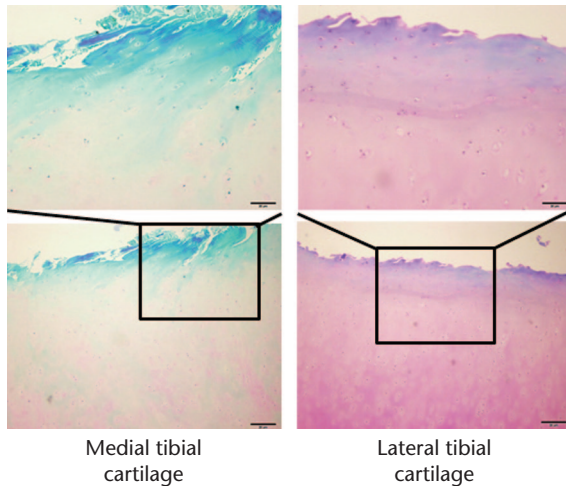


Fig. 1a

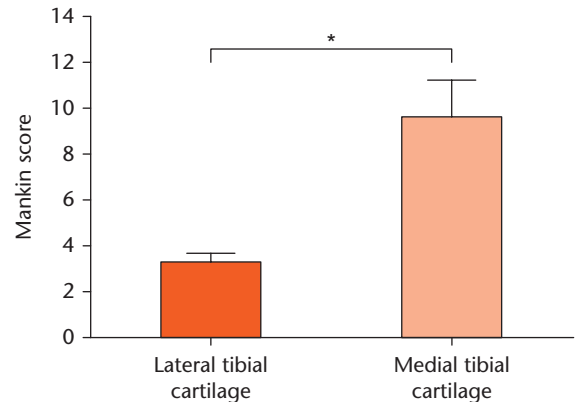


Fig. 1b

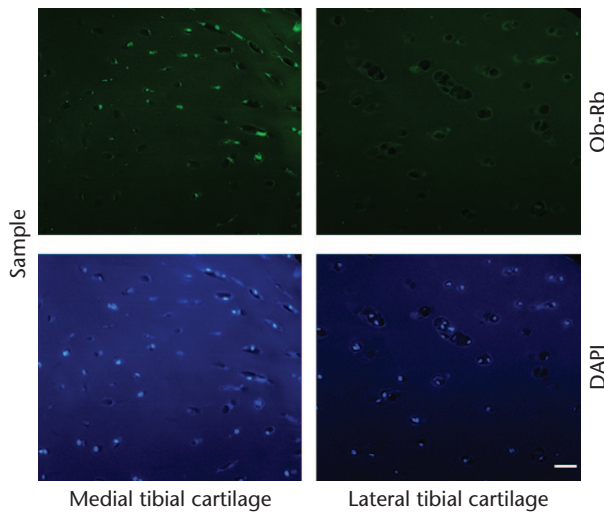


Fig. 1c

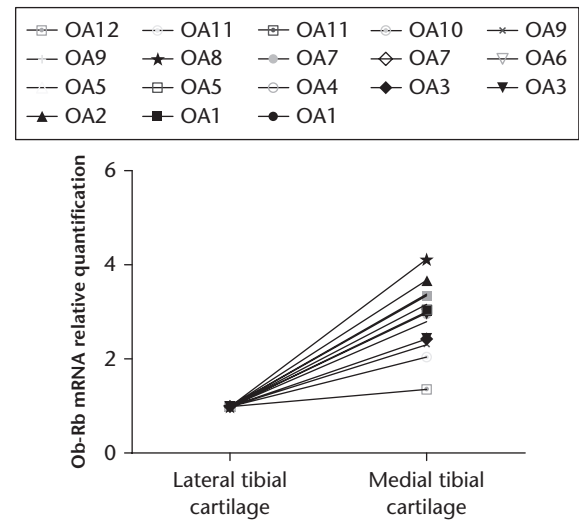


Fig. 1d

Degenerative cartilage with high expression of the long form of the leptin receptor (Ob-Rb). a) Analysis of the degree of lateral and medial tibial cartilage degeneration by using Safranin O and Fast Green staining (scale bar: 20  $\mu$ m). b) Chart showing histological grading as scored using the Mankin scoring system.<sup>16</sup> Error bars represent the mean and standard deviation. c) The results of immunofluorescence revealed distribution of Ob-Rb-positive cells in medial and lateral cartilage samples of the tibial plateau (scale bar: 20  $\mu$ m). d) Chart showing medial and lateral cartilage samples of the tibial plateau were collected from 12 osteoarthritis patients. Ob-Rb messenger RNA (mRNA) expression was detected in medial and lateral cartilage samples of the tibial plateau by reverse transcriptase polymerase chain reaction (RT-PCR). Relative Ob-Rb mRNA expression of each paired group in the medial cartilage sample was normalized to the expression in the lateral cartilage samples. \* $p < 0.05$  was considered statistically significant.

leptin therefore induced senescence in the chondrocytes. High doses of leptin induce senescence by p53/p21 pathway activation in chondrocytes (Figs 2d and 2e).

**After overexpression of Ob-Rb, the physiological dose of leptin induced cell senescence in chondrocytes.** The lateral cartilage of the tibial plateau, as a non OA-affected region, has a low expression of Ob-Rb (Fig. 1a). After performing polymerase chain reaction (PCR) to verify the effect of Ob-Rb overexpression by lenti-Ob-Rb (Fig. 3a), the Ob-Rb-overexpressing chondrocytes and controls were treated with different doses of leptin for two days. The results showed that a physiological dose of leptin activated the p53/p21 pathway in the chondrocytes (Figs 3b and 3c). SA- $\beta$ -gal staining showed that after

overexpression of Ob-Rb, the level of SA- $\beta$ -gal-positive cells significantly increased in the chondrocytes (Fig. 3d). These results showed that the physiological dose of leptin induced cell senescence in Ob-Rb-overexpressing chondrocytes. This finding indicates that a high expression of Ob-Rb in cartilage may cause degeneration by mediating leptin pathway activation.

**High doses of leptin inhibit autophagy by activating the mTOR signalling pathway in chondrocytes.** Western blot analysis revealed that high doses of leptin significantly decreased the levels of autophagy, as measured by flow cytometry (Fig. 4a), while significantly reducing LC3-II expression and increasing p62 expression (Figs 4b and 4c). The lysosomes were dyed with LysoTracker

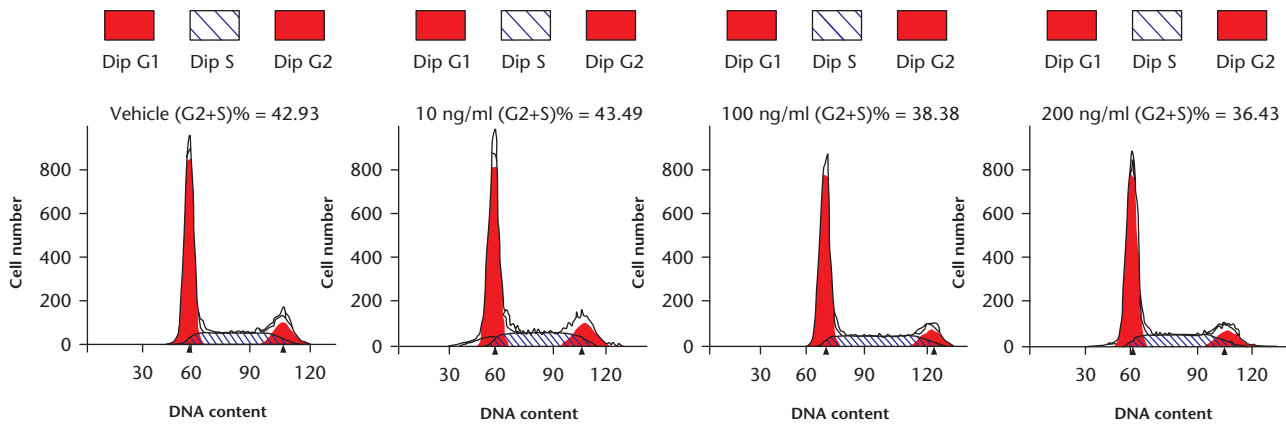


Fig. 2a

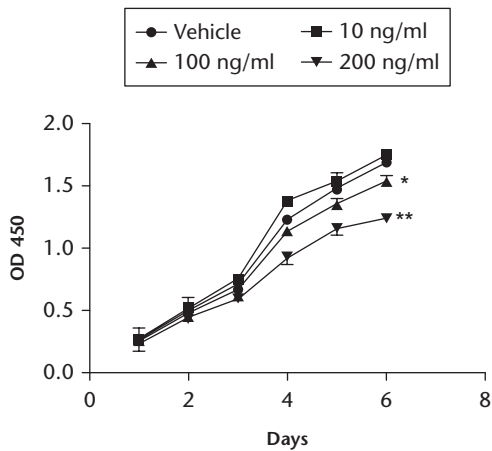


Fig. 2b

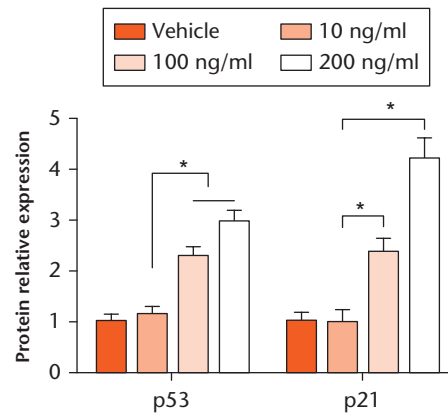


Fig. 2c

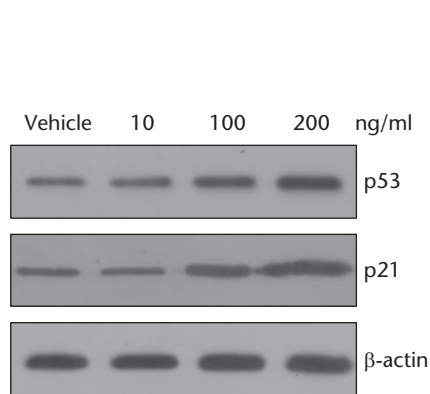


Fig. 2d

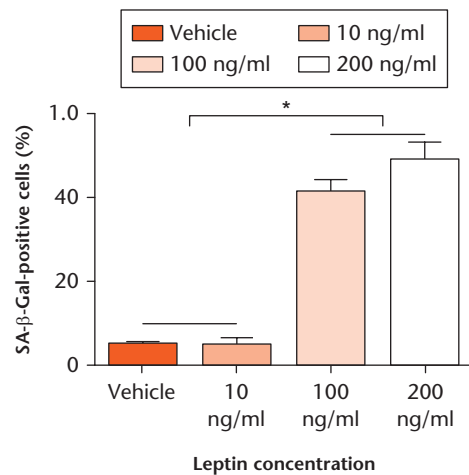


Fig. 2e

High-dose leptin causes chondrocyte senescence. a) Histograms showed chondrocyte cell cycle analysis after different doses of leptin treatment for two days. Compared with vehicle and 10 ng/ml doses of leptin treatment, 100 ng/ml and 200 ng/ml leptin causes chondrocyte cell cycle arrest at phase G1 and decreases the cell proliferation rate by reducing the (G2+S)%. b) Chart showing the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, Maryland) analysis results of cell viability after different doses of leptin treatment. c) Chart showing that high-dose leptin dramatically induces chondrocyte senescence. Relative protein abundance of each blot was normalized to the grey value of  $\beta$ -actin. Error bars indicate the mean and standard deviation. d) The expression of senescence markers p53 and p21 dramatically increased in chondrocytes when treated by high-dose leptin. e) Chart showing senescence cells (senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal)-staining positive cells) increased by high-dose leptin. Error bars indicate the mean and standard deviation. \* $p < 0.05$  was considered statistically significant.

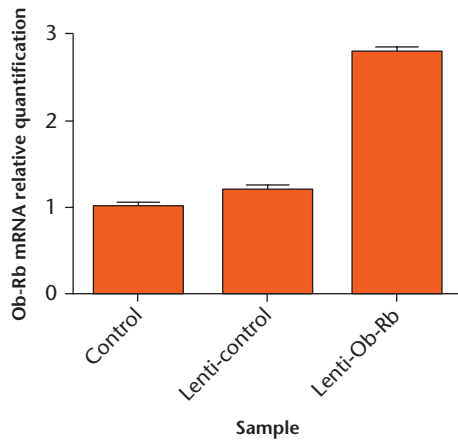


Fig. 3a

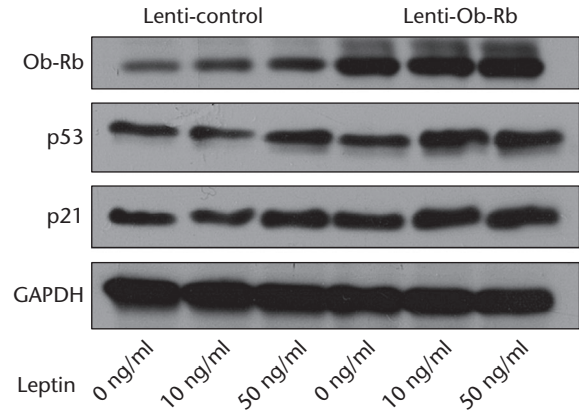


Fig. 3b

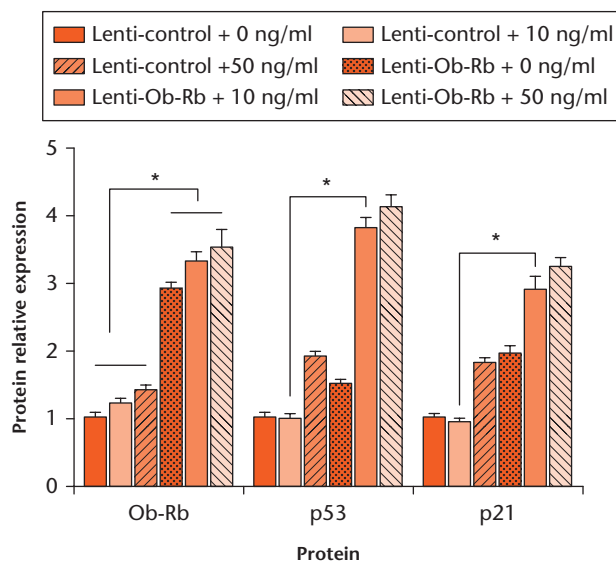


Fig. 3c

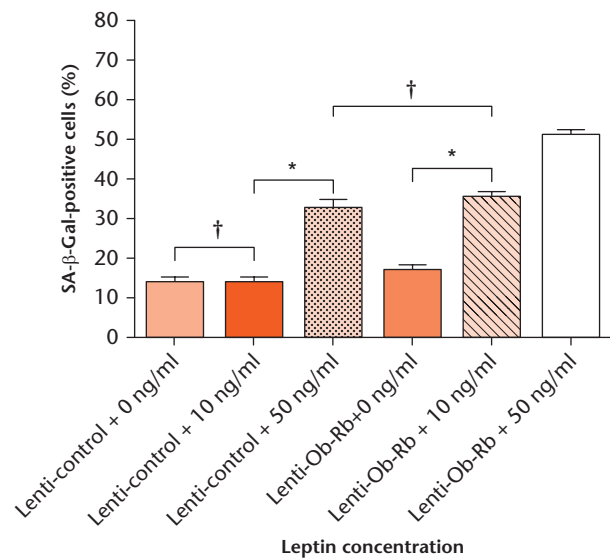


Fig. 3d

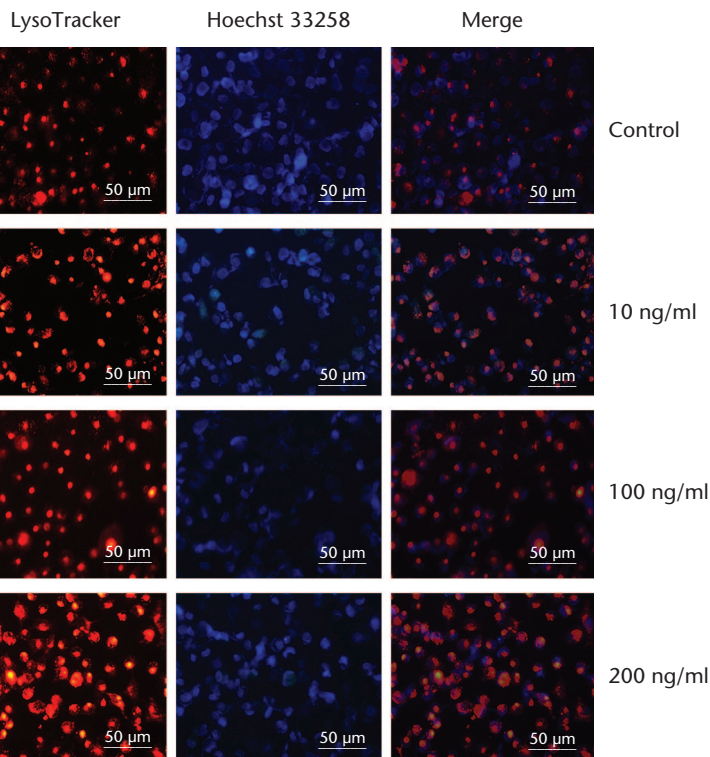
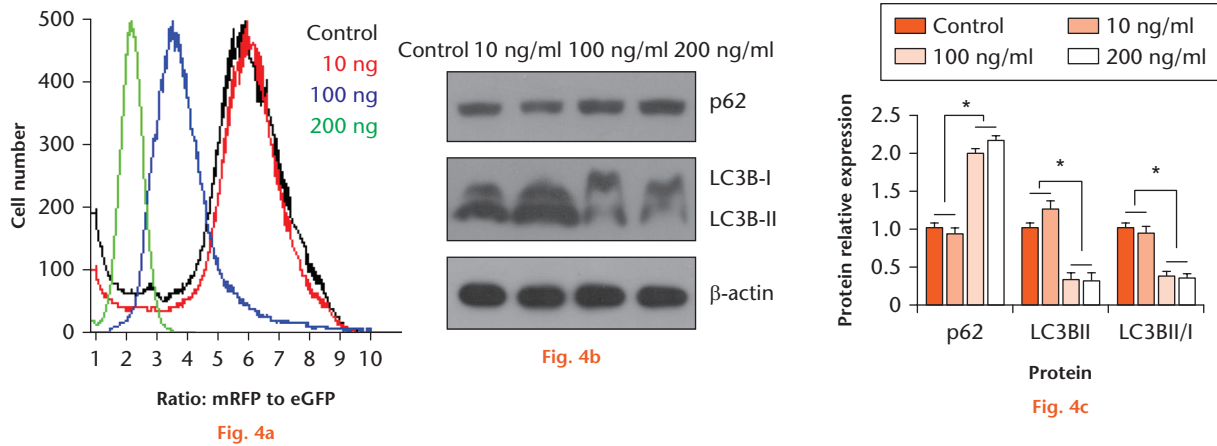
After overexpression of the long form of the leptin receptor (Ob-Rb), the physiological dose of leptin induced cell senescence in chondrocytes. a) Chart showing that after lenti-Ob-Rb, expression of Ob-Rb messenger RNA (mRNA) in chondrocytes derived from the lateral tibial plateau cartilage was detected by real-time polymerase chain reaction (PCR). Relative Ob-Rb mRNA expression of each group was normalized to the expression of GAPDH mRNA. Error bars indicate the mean and standard deviation. b) Chart showing that after Ob-Rb overexpression, the Ob-Rb-overexpressed chondrocytes and control were treated with different doses of leptin for two days. Expression of p53/p21 was then detected and statistically analyzed. c) Chart showing statistical analysis of the expression of p53 and p21. Relative protein abundance of each blot was normalized to the grey value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars indicate the mean and standard deviation. d) Chart showing that after Ob-Rb overexpression, the Ob-Rb-overexpressed chondrocytes and control were treated with different doses of leptin for two days. The senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal)-staining positive cells were counted and statistically analyzed in these samples. Error bars indicate the mean and standard deviation. \* $p < 0.05$  was considered statistically significant. † $p < 0.01$ .

(Beyotime Institute of Biotechnology), and the results revealed that high doses of leptin caused lysosome accumulation, which means that high doses of leptin can inhibit autophagy (Fig. 4d).

The targets of rapamycin (mTOR) play an important role in regulating autophagy in mammals.<sup>19</sup> Immunohistochemistry analysis of the mTOR antibody showed that the areas of cartilage that had degenerated the most also had higher mTOR expression than other areas in the same OA patient (Fig. 5a). We next examined S6K (a downstream target of mTOR signalling) expression.<sup>20</sup> Western blot analysis showed that high doses of leptin

significantly increased the level of phosphorylated S6K (Figs 5b and 5c).

**Blocking the mTOR signalling pathway can restore autophagy and partially reverse senescence induced by leptin in chondrocytes.** To assess the effect of mTOR on the inhibition of autophagy by leptin in chondrocytes, we measured the protein expression of Beclin1, S6K, and pS6K in the presence and absence of rapamycin and AZD8055. However, the leptin-mediated increase in Beclin1 was abolished once mTOR signalling was inhibited by rapamycin or AZD8055, which was demonstrated by diminished levels of phospho-S6K protein (Figs 6a



**Fig. 4d**

High-dose leptin inhibits autophagy in chondrocytes. a) High-dose leptin decreased autophagic flux in chondrocytes. b) Expression of p62 and LC3B-I/II were detected by Western blot analysis. c) Chart showing that high-dose leptin significantly increases expression of p62 and LC3B-I/II expression, which are markers for cell autophagy. Relative protein abundance of each blot was normalized to the grey value of  $\beta$ -actin. Error bars indicate the mean and standard deviation. d) In chondrocytes, lysosome was increased when treated with high-dose leptin (scale bar: 50  $\mu$ m). \* $p < 0.05$  was considered statistically significant. mRFP, monomeric red fluorescent protein; eGFP, enhanced green fluorescent protein.

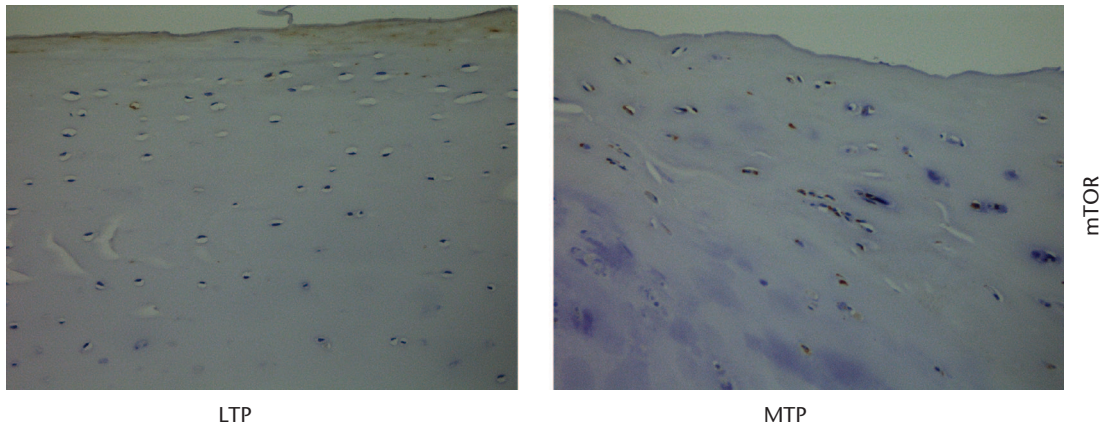
and 6b). Western blot analysis showed that rapamycin or AZD8055 partially reversed the increased expression of p53 and p21 that was induced by leptin in chondrocytes (Figs 6c and 6d). Reduced autophagy levels in the chondrocytes by leptin treatment can be restored by rapamycin or AZD8055 (Fig. 6e). The proportion of SA- $\beta$ -gal-positive chondrocytes induced by leptin was reduced when rapamycin or AZD8055 was present (Fig. 6f). These results suggested that leptin may inhibit autophagy by activating the mTOR pathway in chondrocytes.

Inhibition of the mTOR pathway can restore autophagy and partially reverse leptin-induced senescence in chondrocytes.

## Discussion

Several studies have provided clear evidence supporting the key role of leptin in cartilage homeostasis.<sup>9,21</sup> Studies conducted *in vivo* have shown that injection of leptin into the rat knee joint drives catabolic effects in OA cartilage by increasing the production of MMPs, such as MMP-1,





LTP

MTP

## Sample

Fig. 5a

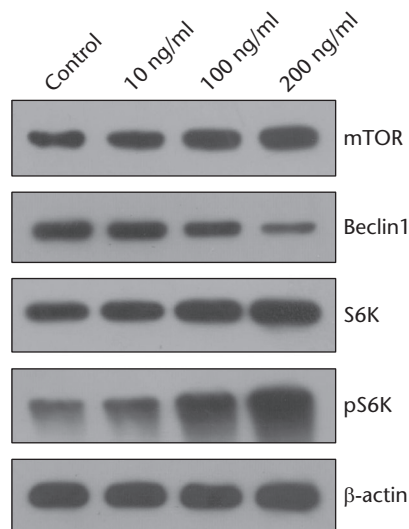


Fig. 5b

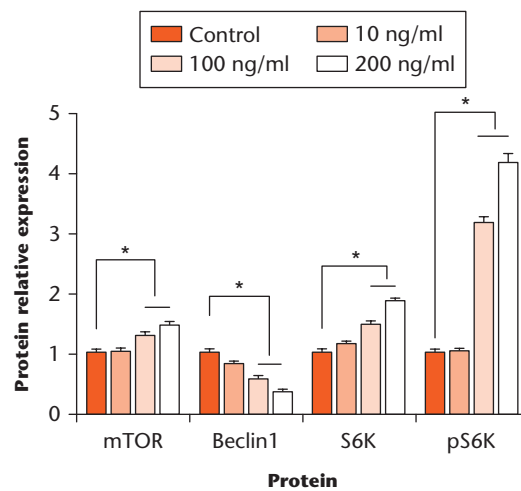


Fig. 5c

High-dose leptin induces senescence and inhibits autophagy by activating the mammalian target of rapamycin (mTOR) pathway. a) Expression and distribution of mTOR in medial tibial plateau (MTP) and lateral tibial plateau (LTP) cartilage areas were detected. b) In chondrocytes treated by high-dose leptin, increased expression of mTOR, S6 kinase (S6K), pS6K, and Beclin1 was detected. c) Chart showing statistical analyses of expression of mTOR, S6K, and pS6K. Relative protein abundance of each blot was normalized to the grey value of  $\beta$ -actin. Error bars indicate the mean and standard deviation. \* $p < 0.05$  was considered statistically significant.

MMP-3, MMP-9, and MMP-13.<sup>7</sup> Furthermore, another study reported a decrease in MMP-13 expression following treatment of cartilage from OA patients with siRNA targeted for leptin.<sup>8</sup> These results confirm a catabolic role for leptin in cartilage homeostasis and an effect of accelerating cartilage degeneration in OA joints.

Our previous research found that CPCs were harvested with characteristics similar to those of the mesenchymal stem cells from rat knee articular cartilage. Leptin alters the fate of differentiating CPCs by inhibiting chondrogenesis and increasing osteogenesis. Additionally, leptin induces senescence in CPCs by activating the p53/p21 pathway and inhibiting the silent mating type information regulation 2 homolog 1 (Sirt1) pathway.<sup>9</sup> This study further confirmed that leptin inhibited cell proliferation (Figs 2a and 2b) and induced cell ageing in chondrocytes

from human OA cartilage (Fig. 2c). We showed that p53 and p21 levels were significantly higher in leptin-treated chondrocytes than in the control (Fig. 2d), indicating that leptin induces senescence by p53/p21 pathway activation in chondrocytes.<sup>22</sup>

We obtained cartilage tissue in the loaded area from the medial and lateral cartilage of the tibial plateau in OA patients and found that the degenerative cartilage (medial cartilage) expressed high levels of Ob-Rb (Fig. 1c). Because this is a self-controlled study, after excluding other factors (including hormones, growth factors, and leptin interference), we believe that the high levels of Ob-Rb may be due to mechanical stress. Despite the lack of radiological data available from earlier in the patient's life, knee varus in patients with OA further increases the stress on the medial cartilage of the tibial plateau. The

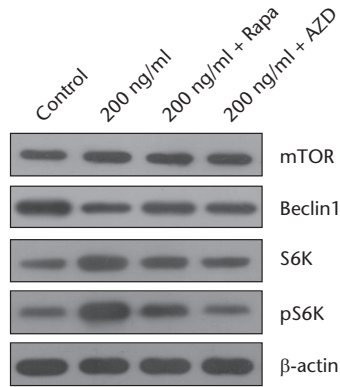


Fig. 6a

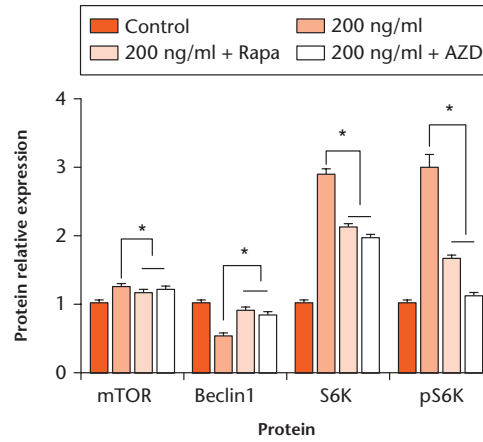


Fig. 6b

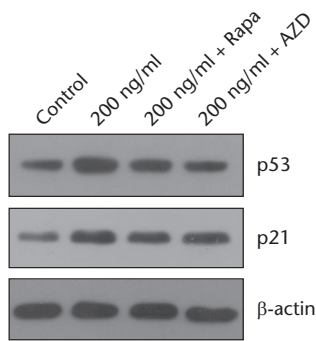


Fig. 6c

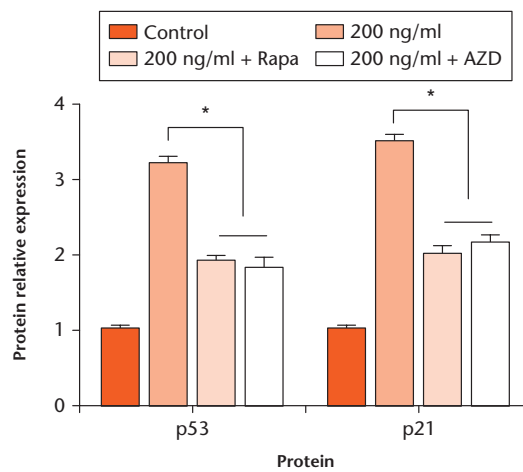


Fig. 6d

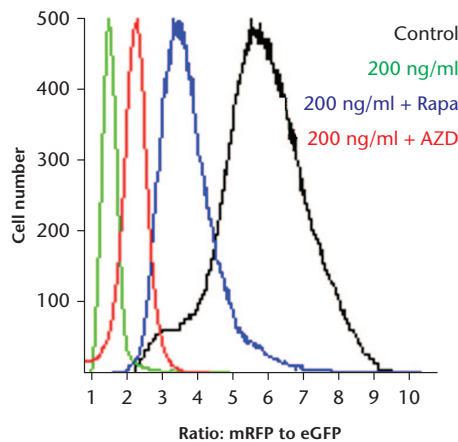


Fig. 6e

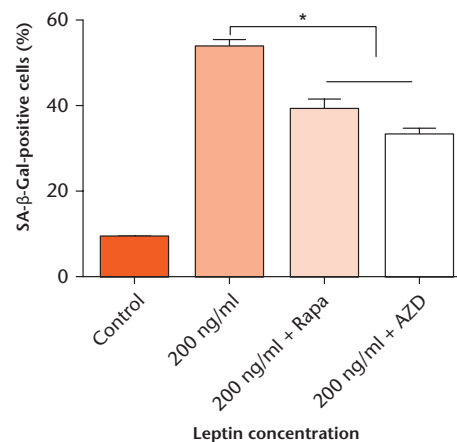


Fig. 6f

Mammalian target of rapamycin (mTOR) inhibitors, rapamycin and AZD8055 can attenuate effects of high-dose leptin on chondrocytes. a) Expression of mTOR, Beclin1, S6 kinase (S6K), and pS6K were detected by rapamycin (Rapa) + 200 ng/ml-leptin and AZD8055 + 200 ng/ml-leptin. b) Chart showing that Rapa and AZD8055 attenuated the effects of high-dose leptin on expression of mTOR, Beclin1, S6K, and pS6K. Relative protein abundance of each blot was normalized to the grey value of β-actin. Error bars indicate the mean and standard deviation. c) Expression of p53 and p21 was detected by Rapa + 200 ng/ml-leptin and AZD8055 + 200 ng/ml-leptin. d) Chart showing that mTOR inhibitors (Rapa and AZD8055) attenuated the effects of high-dose leptin on expression of p53 and p21, which are markers of cell senescence. Relative protein abundance of each blot was normalized to the grey value of β-actin. Error bars indicate the mean and standard deviation. e) mTOR inhibitors partially attenuated the effects of high-dose leptin on chondrocytes' autophagic flux. f) Chart showing that mTOR inhibitors (Rapa and AZD8055) partially attenuated chondrocyte senescence by high-dose leptin induction. Error bars indicate the mean and standard deviation. \*p < 0.05 was considered statistically significant. mRFP, monomeric red fluorescent protein; eGFP, enhanced green fluorescent protein.

weight of obese patients may promote the transcription of Ob-Rb, which was not previously discovered. This finding suggests that obesity may promote the progression of OA through leptin secreted by adipose tissue and that Ob-Rb synthesis caused by body weight synergistically promotes leptin pathway activation.

The loss of normal functionality that results from the induction of senescence has been implicated in age-related diseases and tissue degeneration.<sup>23-25</sup> Mediating leptin-induced senescence in chondrocytes is a therapeutic target for OA. Autophagy can protect human chondrocytes from stress and may be related to the pathogenesis of OA.<sup>11</sup> The results showed that leptin inhibits autophagy and induces senescence. Treating cells with high doses of leptin resulted in a reduction in LC3-II and an increase in p62, showing that leptin inhibits autophagy in chondrocytes (Fig. 4b).

The mTOR, a serine/threonine protein kinase, is a key regulator of the cell growth, metabolism, survival, and lifespan of organisms. mTOR associates with the regulatory-associated protein of mTOR (RAPTOR) and the rapamycin-insensitive companion of mTOR (RICTOR) to form mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).<sup>20</sup> mTOR regulates protein synthesis through the phosphorylation and activation of S6K, and the cartilage-specific deletion of mTOR has been shown previously to upregulate autophagy and protect mice from OA.<sup>26</sup> The results of immunohistochemistry analysis with the mTOR antibody showed that the areas of cartilage with the most severe degeneration have higher mTOR expression than areas of less severe degeneration in the same OA patient (Fig. 5a). However, the levels of mTOR and pS6K in cells treated with high doses of leptin were significantly higher than those in the control (Fig. 5b). In addition, the LC3-II:LC3-I ratio, an autophagy marker, was lower in chondrocytes treated with leptin than in the control, whereas the effect was antagonized by mTOR knockdown. These results demonstrated that leptin inhibits chondrocyte autophagy by modulating the mTOR pathway (Fig. 6b). These findings showed that inhibition of the mTOR pathway can partially reverse leptin-induced senescence in chondrocytes.

García-Prat et al<sup>27</sup> found that re-establishment of autophagy reverses senescence and restores regenerative functions in geriatric satellite cells, suggesting that restoration of autophagy is a therapeutic target of senescence in chondrocytes. Some studies have confirmed that the mTOR/autophagy pathway plays a major role in the development of cartilage degeneration. Zhang et al<sup>26</sup> showed that the genetic loss of mTOR in articular cartilage results in increased autophagy signalling, reduced expression of the OA catabolic factor MMP-13, decreased chondrocyte apoptosis, and significant protection from OA induced by destabilization of the medial meniscus (DMM). The decreased expression of OA

catabolic factors, including MMP-13 and chemokines, suggests the potential of mTOR inhibition in correcting the imbalance between catabolic and anabolic factors during OA.<sup>26</sup> Ito et al<sup>28</sup> showed that selective interference of mTORC1/RAPTOR protects against human disc cellular apoptosis, senescence, and extracellular matrix catabolism with protein kinase B (Akt) and autophagy induction. Zhang et al<sup>29</sup> showed that curcumin promoted autophagy by regulating the Akt/mTOR pathway *in vivo*, suggesting that curcumin-induced autophagy via the Akt/mTOR pathway contributes to the anti-OA effect of curcumin. Wang et al<sup>30</sup> showed that advanced glycation end products (AGEs) can downregulate peroxisome proliferator-activated receptor- $\gamma$  (PPARG) and that PPARG maintains cell viability by activating the Akt/mTOR signalling pathway, as well as by inducing chondrocyte autophagy. Increased autophagy by inhibiting mTOR pathway activation is a therapeutic target for cell senescence, decreased cell function, and the development of cartilage degeneration.

In summary, the present study demonstrated that high doses of leptin induce cell senescence by activating the mTOR pathway in chondrocytes from OA cartilage. Highly expressed Ob-Rb accelerates chondrocyte senescence by activating the leptin pathway in OA.

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#### Author contributions

- X. Zhao: Wrote and revised the manuscript.
- P. Huang: Wrote and revised the manuscript.
- G. Li: Wrote and revised the manuscript.
- Z. Lv: Wrote and revised the manuscript.
- G. Hu: Wrote and revised the manuscript.
- Q. Xu: Wrote and revised the manuscript.
- X. Zhao, P. Huang, G. Li, and Z. Lv contributed equally to this work.

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#### Ethical review statement

- All approved animal experiments were performed in accordance with relevant guidelines and regulations of the Ethics Committee at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China (ethical approval identification number: 2018-143).

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